

Specificity and Function of Archaeal DNA Replication Initiator Proteins

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SUMMARY

Chromosomes with multiple DNA replication origins are a hallmark of Eukaryotes and some Archaea. All eukaryal nuclear replication origins are defined by the origin recognition complex (ORC) that recruits the replicative helicase MCM(2-7) via Cdc6 and Cdt1. We find that the three origins in the single chromosome of the archaeon *Sulfolobus islandicus* are specified by distinct initiation factors. While two origins are dependent on archaeal homologs of eukaryal Orc1 and Cdc6, the third origin is instead reliant on an archaeal Cdt1 homolog. We exploit the nonessential nature of the *orc1-1* gene to investigate the role of ATP binding and hydrolysis in initiator function in vivo and in vitro. We find that the ATP-bound form of Orc1-1 is proficient for replication and implicates hydrolysis of ATP in downregulation of origin activity. Finally, we reveal that ATP and DNA binding by Orc1-1 remodels the protein's structure rather than that of the DNA template.

INTRODUCTION

Several archaeal species have multiple origins of replication per chromosome (Lundgren et al., 2004; Norais et al., 2007; Robinson and Bell, 2007; Robinson et al., 2004, 2007). For example, chromosomes of species in the genus *Sulfolobus* are replicated from three origins (Lundgren et al., 2004; Robinson et al., 2004, 2007). All three origins fire once per cell cycle in all cells (Duggin et al., 2008a). Two of the origins, *oriC1* and *oriC2*, lie adjacent to the genes for the candidate initiator proteins Orc1-1 and Orc1-3, respectively (She et al., 2001). Orc1-1 and Orc1-3 share 35% amino acid sequence identity and are related to both eukaryotic

Orc1 and Cdc6 (Bell and Dutta, 2002; Robinson and Bell, 2007). There is additionally a third Orc1/Cdc6 protein in *Sulfolobus*, Orc1-2, but it is not encoded adjacent to an origin. The third origin, *oriC3*, lies beside the gene for a crenarchaeal-specific protein, WhiP. WhiP is predicted to have a tandem winged-helix structure reminiscent of bacterial plasmid Rep proteins and has sequence similarity to the eukaryal replication factor Cdt1 (Robinson and Bell, 2007). Biochemical DNA footprinting studies have revealed that complex interactions among the *Sulfolobus solfataricus* Orc1/Cdc6 proteins, WhiP, and the replication origins are possible in vitro (Robinson and Bell, 2007; Robinson et al., 2004, 2007). More specifically, Orc1-1, Orc1-2, and WhiP bind to all three origins, and Orc1-3 binds to *oriC2* and *oriC3*. However, the precise requirements for origin function either in vivo or in vitro remain unknown. The Orc1 proteins are AAA+ proteins that undergo a single-turnover hydrolysis of ATP to ADP, but the functional consequences of ATP binding and hydrolysis are not yet known (Singleton et al., 2004). In the current work, we address these fundamental issues. First, we determine the initiator protein requirements for origin function in vivo in the genetically tractable strain *Sulfolobus islandicus* (Sis) (Guo et al., 2011; Zhang et al., 2010). We find that none of the Orc1/Cdc6 genes are essential for cell viability. Furthermore, we reveal that while *oriC1* requires the *orc1-1* gene product for function, *oriC2* is dependent on Orc1-3. Finally, we find that *oriC3* functions independently of all three Orc1/Cdc6 proteins but requires the Cdt1-related protein WhiP.

Next, we exploit the nonessential nature of Orc1-1 to express mutant forms of the protein and assay their phenotype with respect to *oriC1* firing in vivo. Our data reveal that expression of a Walker B mutant form of the protein that can bind but not hydrolyze ATP leads to an overreplication phenotype, with elevated levels of initiation at *oriC1*. We further observe an enhanced ability of the Walker B mutant protein to recruit MCM to origin DNA in vitro. Finally, we demonstrate that the ATP-bound form of the protein, when bound to DNA, does not

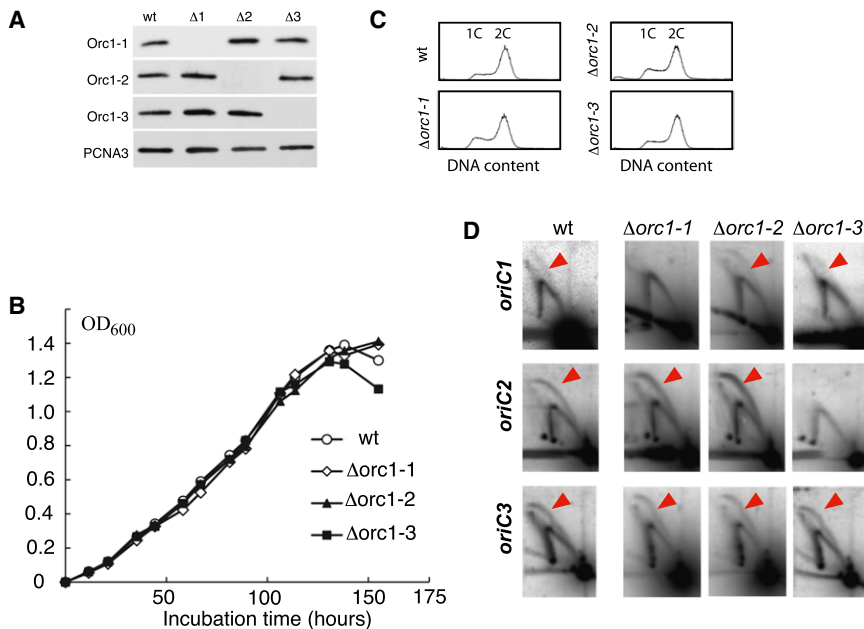


Figure 1. Individual Orc1/Cdc6 Genes Are Nonessential

(A) Western analyses of Orc1/Cdc6 levels in wild-type (wt) and strains deleted for *orc1-1* ($\Delta 1$), *orc1-2* ($\Delta 2$), or *orc1-3* ($\Delta 3$). Antiserum against PCNA3 was used as a loading control.

(B) Growth curves of the indicated strains, following growth by absorbance at 600 nm.

(C) Flow-cytometry profiles of WT and mutant strains. Positions of single- and double-chromosome (1C and 2C) content cells are indicated.

(D) 2D gel analysis of origin activity in wild-type (wt) and the indicated mutant strains. The position of the bubble arc, indicative of replication initiation, is indicated by a red arrowhead.

See also Figures S5, S7, and Tables S1, S2, S3, and S4.

impinge on the geometry of the DNA but remodels its own conformation.

RESULTS

None of the *orc1/cdc6* Genes Are Essential for Viability

To address the roles of the individual Orc1/Cdc6 paralogs, we generated mutant *S. islandicus* cell lines in which the relevant genes were deleted (Figure 1A). The resultant strains were viable and showed growth and flow-cytometry profiles that were essentially indistinguishable from the wild-type (WT) strain (Figures 1B and C). We assayed origin activity using two-dimensional (2D) neutral-neutral agarose gel electrophoresis. As can be seen in Figure 1D, deletion of *orc1-1* abrogates firing of *oriC1* but does not affect initiation at *oriC2* or *oriC3*. Deletion of *orc1-2* does not affect firing at any of the three origins, but deletion of *orc1-3* abolishes *oriC2* activity.

Next, we constructed double mutants lacking pairs of Orc1/Cdc6 proteins (Figure 2A). All three possible double mutants were generated, but attempts to generate a triple mutant were unsuccessful. The growth rates of all three double mutants were lower than WT or single mutants, and flow cytometry suggested an elevated S phase population of cells in the $\Delta orc1-2/\Delta orc1-3$ and $\Delta orc1-1/\Delta orc1-3$ strains (Figures 2B and 2C). The strain lacking both Orc1-2 and Orc1-3 phenocopies the single *orc1-3* deletion, with no firing detectable at *oriC2* but retaining *oriC1* and *oriC3* activity (Figure 2D). Similarly, as observed for deletion of *orc1-1* alone, deletion of both *orc1-1* and *orc1-2* inactivated *oriC1*, but *oriC2* and *oriC3* still fired. (Figure 2D). Significantly, the $\Delta orc1-1/\Delta orc1-3$ double mutant only showed replication initiation at *oriC3*; no replication initiation activity was detected at either *oriC1* or *oriC2* in this strain (Figure 2D).

To exclude the possibility that hitherto-undetected cryptic origins might have been activated in the mutant backgrounds,

we determined the genome-wide replication profile of the WT and $\Delta orc1-1/\Delta orc1-3$ mutant strains by marker frequency analysis (MFA) in conjunction with next-generation sequencing. In agreement with previous studies, three peaks can be observed in the WT strain, corresponding to origin activity at *oriC1*, *oriC2*, and *oriC3* (Figure 2E). As previously seen, the peak at *oriC2* is of lower amplitude due to the broader temporal window of firing of this origin compared to the other two. Significantly, only a single peak is observed in the $\Delta orc1-1/\Delta orc1-3$ mutant strain, revealing that the chromosome of this strain is replicated from a single origin, *oriC3* (Figure 2F). There is a modest shoulder on the slope of the peak coincident with the position of *oriC1*, but potential firing here is clearly at a very low level, undetectable by 2D gel analyses and independent of Orc1-1 or Orc1-3. We further note that the trough corresponding to replication termination events is repositioned in this strain to 180° around the chromosome from *oriC3*.

The *whiP* Gene Is Required for *oriC3* Function

Interestingly, none of the individual or double Orc1/Cdc6 gene deletions impinged upon firing of *oriC3*. We therefore speculated that this origin could be functioning independently of Orc1/Cdc6 paralogs. Notably, the Cdt1-related protein WhiP is encoded adjacent to *oriC3* (Robinson and Bell, 2007). Further, we have previously shown that this protein binds to sequence motifs in the *oriC3* region, immediately downstream of the *whiP* gene (Robinson and Bell, 2007). We therefore generated a strain lacking WhiP by introducing tandem stop codons following the start codon of the open reading frame (ORF). The absence of WhiP in the resultant line was confirmed by western blotting (Figure 3A). Cell growth and flow cytometry revealed that the mutant cells grew slower than either WT cells or any of the single *orc1* deletion mutants and with a 2-fold increase in sub-1C signal in the flow cytometry (Figures 3B and 3C). Significantly, loss of the WhiP protein correlated with loss of firing at *oriC3* but had no effect on initiation at *oriC1* and *oriC2*, as adjudged both by 2D neutral-neutral agarose gel electrophoresis analysis and MFA (Figures 3D and 3E). Thus, while *oriC3* is independent of

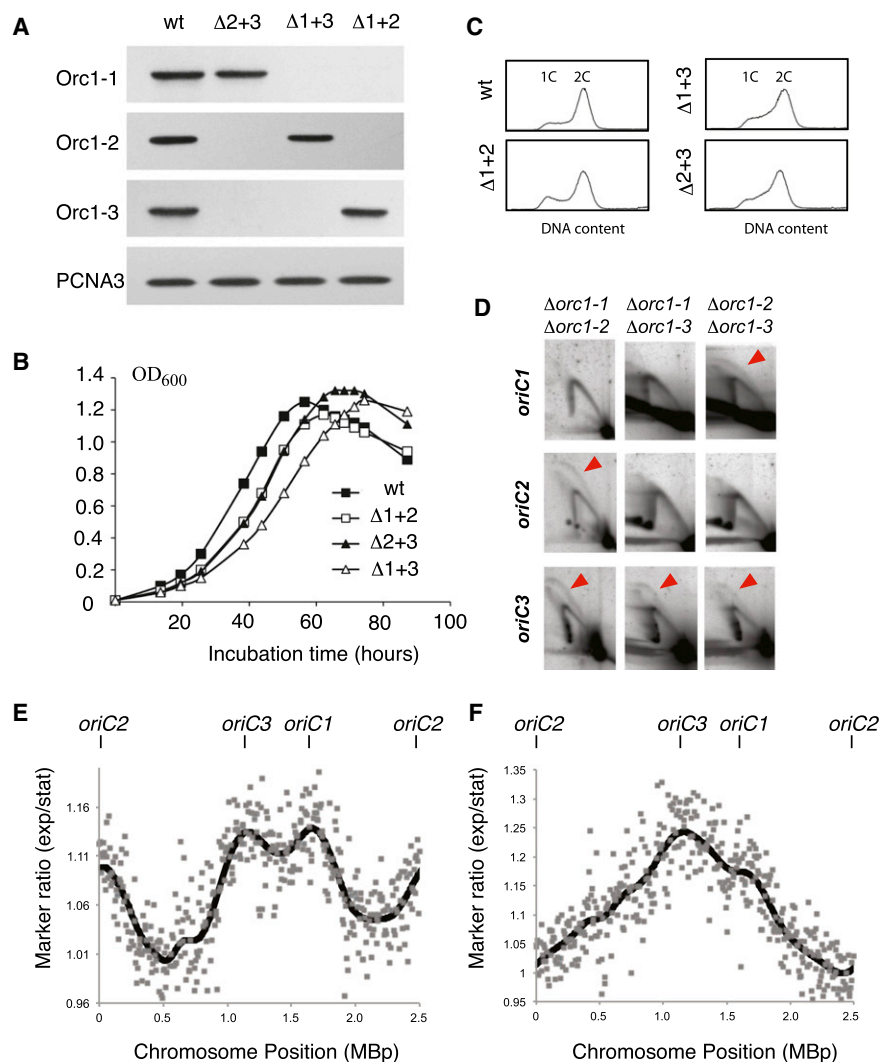


Figure 2. Effect of Deleting Combinations of Orc1/Cdc6 Genes

(A) Western analyses of Orc1/Cdc6 levels in wild-type (wt) and strains deleted for *orc1-2* and *orc1-3* ($\Delta 2+3$), *orc1-1* and *orc1-3* ($\Delta 1+3$), or *orc1-1* and *orc1-2* ($\Delta 1+2$). Antiserum against PCNA3 was used as a loading control.

(B) Growth curves of the indicated strains, following growth by absorbance at 600 nm.

(C) Flow-cytometry profiles of WT and mutant strains. Positions of single- and double-chromosome (1C and 2C) content cells are indicated.

(D) 2D gel analysis of origin activity in WT (wt) and the indicated mutant strains. The position of the “bubble arc,” indicative of replication initiation, is indicated by a red arrowhead.

(E and F) Marker frequency analysis of wild-type cells (E) and cells lacking Orc1-1 and Orc1-3 (F). Marker ratios of sequence tag abundance across the chromosome of exponentially growing cells normalized to nonreplicating stationary phase cells are plotted relative to genome position. The locations of the three origins are indicated above the plots.

See also Figure S7, and Tables S1, S2, S3, and S4.

individual and pairwise combinations of Orc1/Cdc6 proteins (Figures 1 and 2), WhiP is required for replication initiation at this origin (Figures 3D and 3E).

Binding of Initiators to Origins In Vivo

To address origin occupancy by the initiator proteins in vivo, we performed chromatin immunoprecipitation using antisera generated against Orc1-1, Orc1-2, and Orc1-3 and WhiP. In agreement with the lack of requirement for Orc1-2 for firing of any origin, we did not detect binding of Orc1-2 to any of the origins. Also in good agreement with the genetic analyses, we find that *oriC1* is bound by Orc1-1, *oriC2* shows strongest association with Orc1-3, and while no association of any Orc1/Cdc6 protein is observed at *oriC3*, the WhiP protein is specifically localized at this origin (Figures 4A–4D).

Archaea are believed to undergo coupled transcription and translation (French et al., 2007). We were, therefore, concerned that the ability to chromatin immunoprecipitate DNA corresponding to the origins located adjacent to the relevant initiator protein gene could reflect crosslinking of nascent poly-

peptides to the initiator ORF rather than to the origin itself. We therefore performed chromatin immunoprecipitation sequencing (ChIP-seq) analyses of the three Orc1/Cdc6 proteins and WhiP to give a higher-resolution analysis of the proteins' DNA occupancy. As can be seen in Figures 4E–4G and Figure S1, the relevant proteins showed strong association with the origin loci. Notably, the WhiP ChIP recovers most DNA at internal positions within the WhiP ORF.

In addition, a broad but shallow peak was observed over the previously described in vitro binding sites (Robinson and Bell, 2007). These lie at the boundaries of the downstream intergenic region, to which initiation at *oriC3* has been mapped (Robinson et al., 2007). The *whiP* ORF contains conserved AT-rich sequence repeats (Figure S1) and footprinting studies reveal that the protein binds to these with nanomolar affinity. We reiterate that the *whiP* mutation that we employed to inactivate the gene for the genetic studies was by introduction of stop codons at the start of the ORF, rather than by deletion of the ORF (and thus its internal repeats).

In agreement with the 2D gel analyses, we detect Orc1-3 binding at *oriC2* but do not observe significant binding of Orc1-1 to this locus. This was surprising to us because previous ChIP analyses and biochemical and structural studies of the closely related organism, *Sulfolobus solfataricus* (Sso), had demonstrated that both Orc1-1 and Orc1-3 bind to *oriC2* in that species (Dueber et al., 2007, 2011; Robinson et al., 2004). However, examination of the sequence of *S. islandicus oriC2* reveals that one of the characterized mini origin recognition

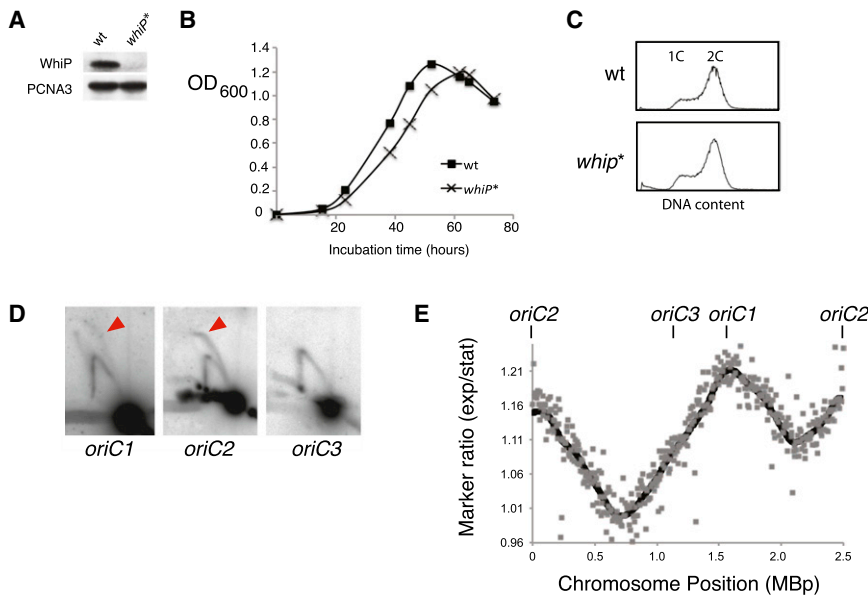


Figure 3. WhiP Mediates Replication Initiation at *oriC3*

(A) Western analyses of WhiP in the wild-type (wt) strain and a strain containing a version of *whiP* with tandem stop codons following the start codon (*whiP**).

(B) Growth curves of the indicated strains, following growth by absorbance at 600 nm.

(C) Flow-cytometry profiles of WT and mutant strains. Positions of single- and double-chromosome (1C and 2C) content cells are indicated.

(D) 2D neutral-neutral agarose gels showing *oriC1*, *oriC2*, and *oriC3* activity in the *whiP** mutant strain. The position of the bubble arc, indicative of replication initiation, is indicated by a red arrowhead.

(E) MFA profile of the *whiP** mutant. The positions of the three origins are shown.

See also Figures S6, S7, and Tables S1, S2, S3, and S4.

box (mORB) Orc1-1 binding sites has several base substitutions in conserved positions in the binding site when compared with *S. solfataricus oriC2* (Figure S2). Additionally, the second mORB element has substitutions immediately adjacent to the core consensus motif. We therefore tested the *S. islandicus* Orc1-1 and Orc1-3 proteins' DNA binding preferences in vitro. Remarkably, but in agreement with the ChIP analyses, Sis Orc1-1 binds with at least 10-fold lower affinity than Sso Orc1-1 to either of the mORB-like elements in Sis *oriC2*. Notably, Sis Orc1-1 has lower affinity for Sso *oriC2* than has Sso Orc1-1. As expected from the ChIP studies, Orc1-3 binds to Sis *oriC2*-derived sequences (Figure S2). Thus, both origin sequence and initiator protein sequence define the differential binding of Orc1-1 to *oriC2* between *S. solfataricus* and *S. islandicus*. Remarkably, even between two closely related species within the *Sulfolobus* genus, we can observe variation within initiator protein specificity and function.

Nucleotide Binding and Orc1-1 Function at *oriC1* In Vivo

We wished to exploit the nonessential nature of Orc1-1 to perform structure-function analyses in living cells. We therefore sought to complement the chromosomal Orc1-1 deletion with a plasmid-encoded copy. Initially, we expressed the gene from its own promoter on a low-copy-number plasmid. We could detect Orc1-1 protein by western blotting (Figure 5A), and the protein localized to *oriC1* as adjudged by ChIP experiments (Figure 6B). However, we were unable to detect any replication initiation at *oriC1* by neutral-neutral 2D agarose gel electrophoresis (Figure 5B). We therefore expressed Orc1-1 from a strong arabinose-inducible promoter (Albers et al., 2006). Either basal or induced expression of the protein from the *araS* promoter led to initiation at *oriC1* as detected by 2D gels (Figure 5B). Indeed, the intensity of the "bubble" arc initiation structure was reproducibly stronger than the rather weak arc observed in WT cells (Figure 5B). This enhanced intensity prompted us to evaluate the DNA content of these cells. Interestingly, cells that

express the Orc1-1 protein from the arabinose promoter in either basal or induced conditions show an aberrant flow-cytometry profile with an elevated population of cells, with between 1C and 2C and greater than 2C genome contents (Figure 5C). Furthermore, fluorescence microscopy of these cells reveals altered cell morphologies, with 14% of cells extruding vesicle-like structures (Figure 5D).

Transcription of the *orc1-1* gene in *S. acidocaldarius* and *S. solfataricus* is known to be regulated following perturbation of the cell cycle and after a number of cellular stresses (Fröls et al., 2007; Götz et al., 2007; Lundgren and Bernander, 2007; Ortmann et al., 2008). We therefore wished to determine the profile of expression of this and the other initiator protein genes during a minimally perturbed cell cycle. We employed our previously described "Baby machine" method for synchronizing cells (Duggin et al., 2008a). For these studies, we utilized *S. acidocaldarius* cells because they have given the best synchrony in these experiments. We observe a striking cyclic pattern of transcript levels for *orc1-1*, *orc1-3*, and *whiP* (Figure 5E), although we have previously demonstrated that Orc1/Cdc6 protein levels remain constant across the cell cycle (Duggin et al., 2008a). Nevertheless, all three transcripts show lowest levels in S phase and peak in or just before G1 phase. Interestingly, *orc1-3* mRNA levels peak slightly later than *orc1-1*, correlating with the broader temporal window of *oriC2* firing (Duggin et al., 2008a).

The Orc1/Cdc6 proteins possess a AAA+ domain and previous work has shown that this catalyzes a single turnover ATP hydrolysis event, resulting in stable binding of ADP (Singleton et al., 2004). Indeed, Orc1/Cdc6s from a range of archaeal species, when purified as recombinant proteins from bacteria, are isolated exclusively in the ADP-bound form. By analogy with bacterial DnaA, we assumed that the ATP-bound form of Orc1/Cdc6 proteins is the active form (see also below) (Kaguni, 2011; Mott and Berger, 2007). We therefore speculated that the cell-cycle-dependent wave of *orc1-1* transcription

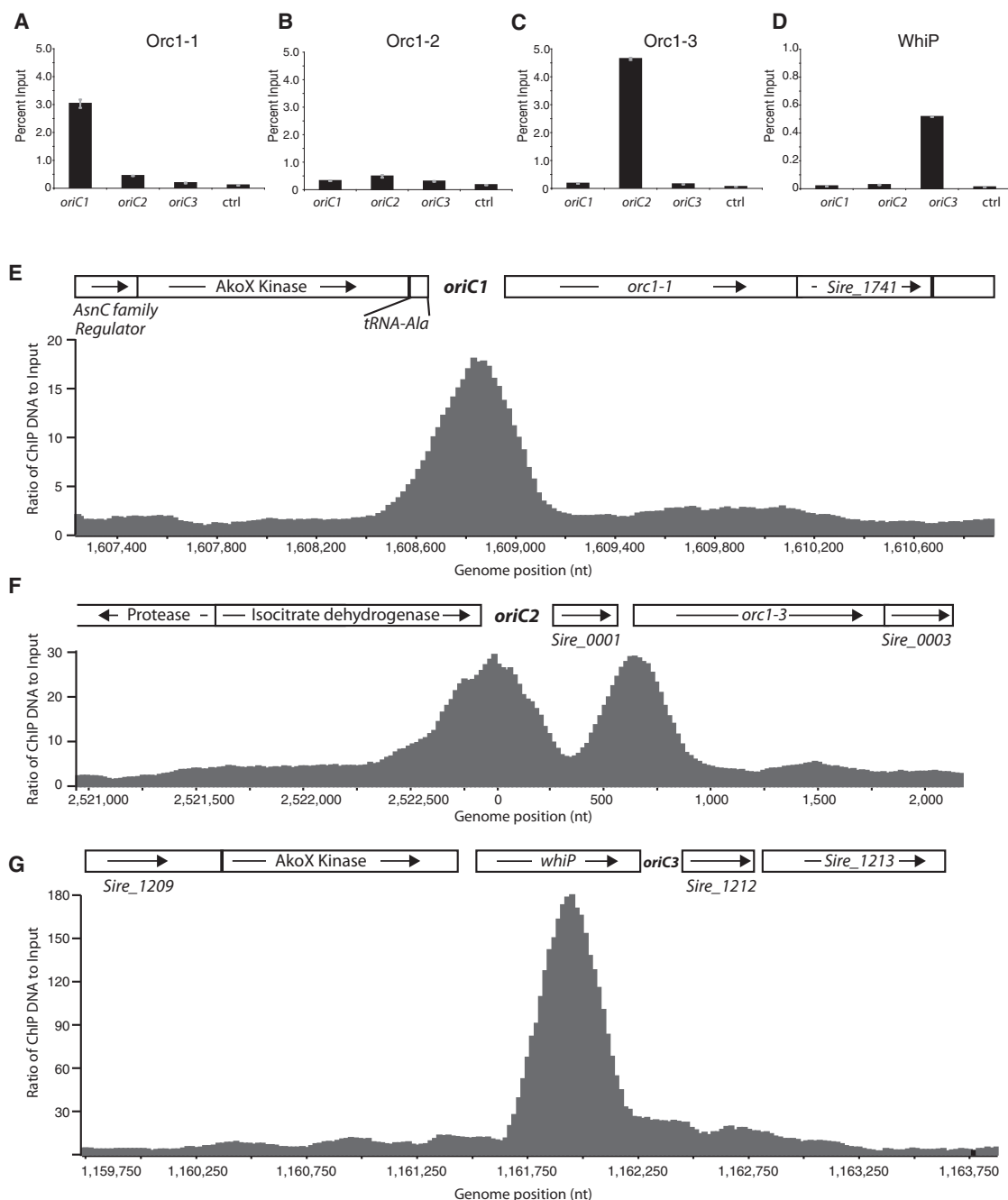


Figure 4. Origin Binding In Vivo by the Orc1/Cdc6 Proteins and WhiP

(A–D) ChIP analyses to assess the binding of the indicated initiator proteins to the three origin loci in vivo. The percent of input DNA recovered was calculated for each origin locus and a distal control locus (ctrl, *Sire2328*). Bars represent the SD calculated from the triplicate experiments.

(E–G) ChIP-seq results with Orc1-1, Orc1-3, and WhiP antisera, respectively. Genome coordinates are shown below each panel and the location of each origin indicated in bold. See also Figure S1. The y axis scale is the value obtained when normalizing ChIP read counts to input DNA.

See also Figure S2, and Tables S1, S2, S3, and S5.

would result in transiently increased levels of Orc1-1•ATP and that this could create a permissive window for replication initiation. Expressing Orc1-1 constitutively during the cell cycle from the arabinose promoter would result in temporally ectopic Orc1-1•ATP and could thus account for the overreplication

phenotype observed. To test this, we created mutant derivatives of Orc1-1 with alterations in the Walker A and Walker B motifs. The Walker A K69A is anticipated to be unable to bind ATP and the Walker B E147A mutant should bind but fail to hydrolyze ATP. These predicted properties were confirmed using purified

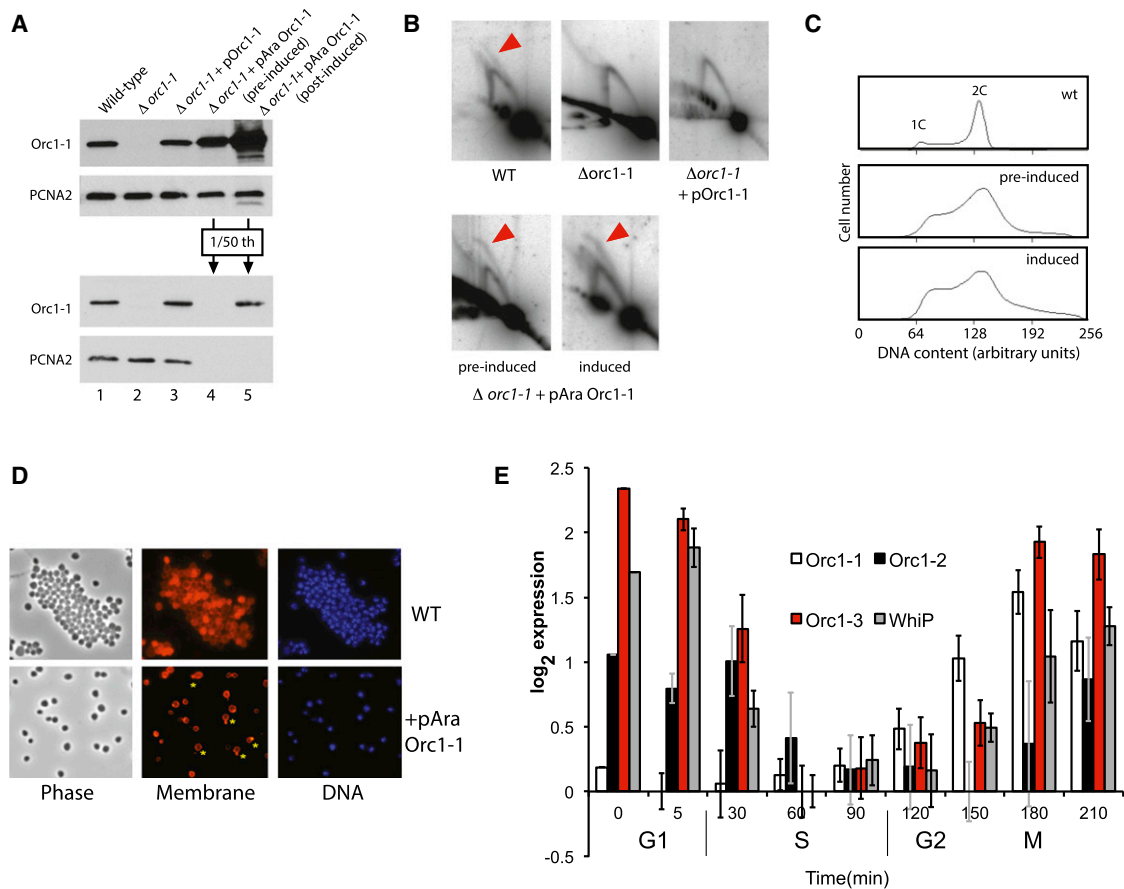


Figure 5. Constitutive Expression of Orc1-1 Causes an Overreplication Phenotype

(A) Western analysis of Orc1-1 levels in WT cells (lane 1), $\Delta orc1-1$ cells (lane 2), the $\Delta orc1-1$ strain complemented with a plasmid encoding the gene for Orc1-1 under the control of its native promoter (lane 3), or under the control of the arabinose promoter under noninducing and inducing conditions (lanes 4 and 5). PCNA2 serves as a loading control. Note that in the lower panels the samples in lanes 4 and 5 contain 1/50th the amount of material present in lanes 1–3.

(B) 2D gel analyses of *oriC1* in the indicated strains. The red arrowheads indicate structures arising from replication initiation.

(C) Flow-cytometry analyses of the DNA content of the indicated strains. Peaks corresponding to 1C and 2C cells are indicated.

(D) Micrographs of the indicated strains. Phase contrast, cells stained with the membrane dye FM4-64X (red), and nucleoids stained with DAPI (blue) are shown. Yellow asterisks indicate cells extruding vesicle-like structures.

(E) Transcript abundance profiles of *orc1-1*, *orc1-2*, *orc1-3*, and *whiP* as a function of position in the cell cycle. Cells were synchronized using a baby machine and RNA purified as described (Samson et al., 2011). Bars are \pm SD.

See also Figures S3, S4, S7, and Tables S1, S2, and S4.

recombinant proteins (Figure S3). To exclude the possibility of compounding effects due to overexpression, the proteins were then expressed from a plasmid using the native *orc1-1* promoter in the $\Delta orc1-1$ strain (Figure 6A). Despite being expressed and binding to *oriC1* as detected by ChIP, the Walker A mutant failed to support detectable initiation at *oriC1* (Figures 6A–6C). In contrast, the Walker B mutant shows a bubble arc of greater intensity than seen in WT cells (Figure 6C). Furthermore, this strain shows an aberrant flow cytometry profile, with 22% of the population with greater than 2C genome content (Figure 6D). Additionally, microscopy reveals the presence of large cells with elevated DNA content; 13% of Orc1-1 E147A-expressing cells are greater than 1.5 μ m in diameter, compared with less than 1% of WT (Figures 6E and 6F). Interestingly, microscopy of the population expressing the Orc1-1 Walker A mutant also shows

aberrant large cells (3.8%); however, in agreement with the flow-cytometry profiles, there is not a discernable population of cells with elevated DNA content (as adjudged by DAPI staining). Taken together, our data therefore indicate that the ATP-bound form of Orc1-1 is proficient for origin firing and, furthermore, implicates the hydrolysis of ATP in regulating origin activity.

Biochemical Consequences of ATP Binding by Orc1-1

To investigate the molecular basis of the distinct properties of the proteins, we first performed gel-filtration analyses. These revealed an increase in retention time of the E147A protein compared to WT, suggestive of a conformational alteration (Figure 7A). Next, we performed analytical ultracentrifugation (AUC) with the WT and E147A proteins (Figure 7B; Figure S3). The AUC revealed that the WT protein migrated in two populations, both

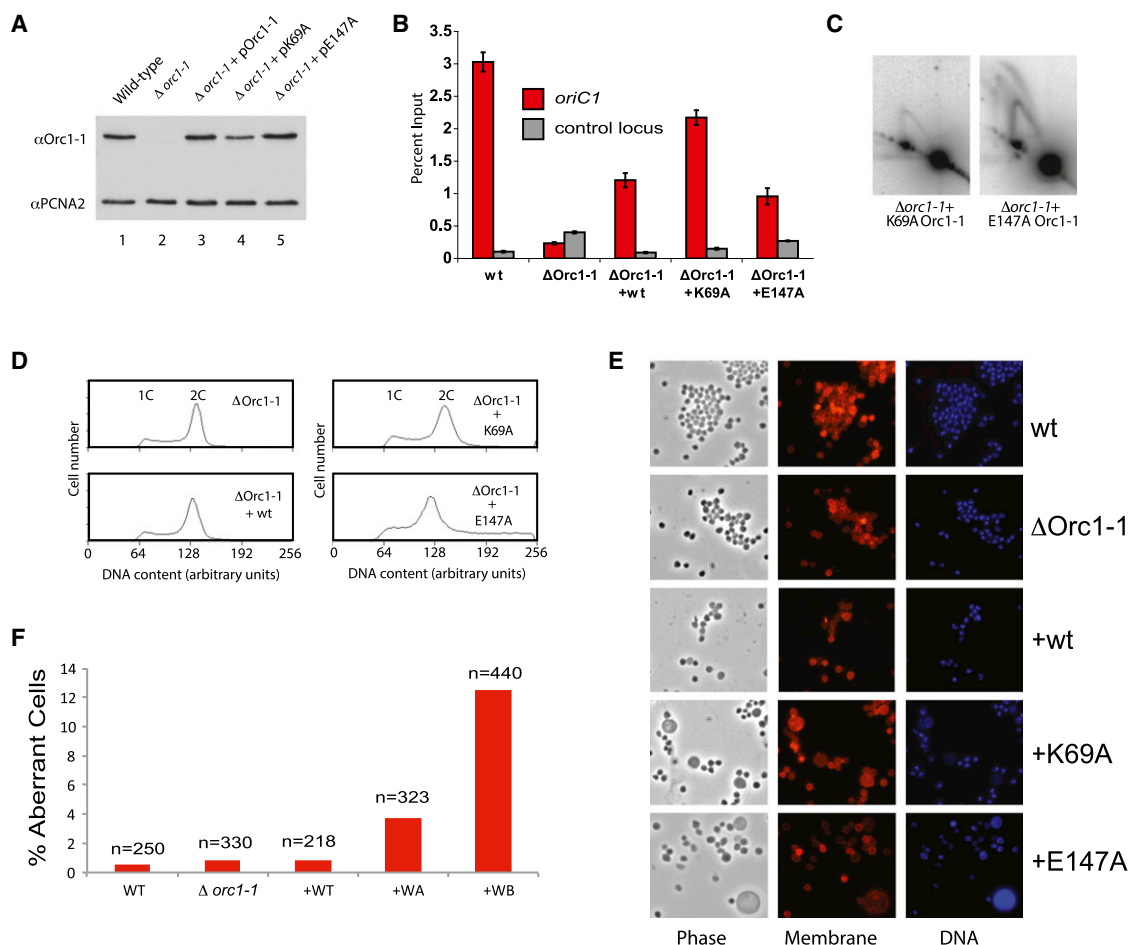


Figure 6. Expression of a Walker B Mutant Orc1-1 Causes an Overreplication Phenotype

(A) Western analysis of Orc1-1 levels in WT (lane 1) and Δ orc1-1 (lane 2) strains and the latter strain complemented with a plasmid encoding the gene for WT Orc1-1 (lane 3), K69A Walker A mutant (lane 4), and E147A Walker B mutant (lane 5) under the control of the native *orc1-1* promoter.

(B) ChIP analysis of the indicated Orc1-1 protein binding to *oriC1* and a distal control locus (*Sire_2328*) in vivo. Error bars are \pm SD.

(C) 2D gel analyses of replication initiation at *oriC1* in the indicated strains. Red arrowheads indicate structures arising due to replication initiation.

(D) Flow-cytometry analyses of the cell-cycle profile of the indicated strains. Positions of 1C and 2C populations are indicated.

(E) Micrographs of the indicated strains (+wt = Δ orc1-1 + pOrc1-1, +K69A = Δ orc1-1 + pOrc1-1 [K69A]; +E147A = Δ orc1-1 + pOrc1-1 [E147A]). Phase contrast, membrane dye FM4-64X (red), and DAPI-stained (blue) images are shown.

(F) Quantitation of aberrant cells (scored as having a diameter greater than 1.5 μ m). Note that large cells in the Walker A strain appear to have less DNA than the large cells in the Walker B strain.

See also Figures S3, S4, S7, and Tables S1, S2, and S4.

corresponding to monomer with distinct conformations. More specifically, 8.3% of the population had a sedimentation coefficient of 2.8 s and frictional ratio of 1.37, revealing a more extended configuration than the majority of the protein that resolved in a peak at 3.7 s with a frictional ratio of 1.07 and thus was more globular. Interestingly, very different behavior was observed with E147A. Although 65% of the E147A protein behaved as globular monomer (frictional ratio of 1.03), there was no peak corresponding to the extended monomer configuration. However, E147A generated a peak with a mass compatible with the formation of a dimer. Furthermore, the sedimentation coefficient and frictional ratio of the monomeric form of the E147A protein were lower than those of the majority WT species, indicating that it adopted a distinct conformation.

Structural studies have revealed that the ADP-bound forms of Orc1/Cdc6 proteins make bidentate interactions with DNA, binding via the C-terminal wH domain and the initiator-specific motif (ISM) in the AAA+ domain. To date, there is no structure available of the ATP-bound form of Orc1/Cdc6 on DNA. However, Wigley and colleagues were successful in denaturing Orc1-2 from *Aeropyrum pernix* (Ape), removing ADP and refolding in the presence of the nonhydrolyzable ATP analog, ADP-NP, prior to crystallization and structure determination (Singleton et al., 2004). The resultant structure revealed a significant alteration in the relative positioning of the wH and AAA+ domain compared to the various conformations adopted by the ADP-bound form (Singleton et al., 2004). Ape Orc1-2 has only 22% sequence identity to *S. islandicus* Orc1-1 and belongs to

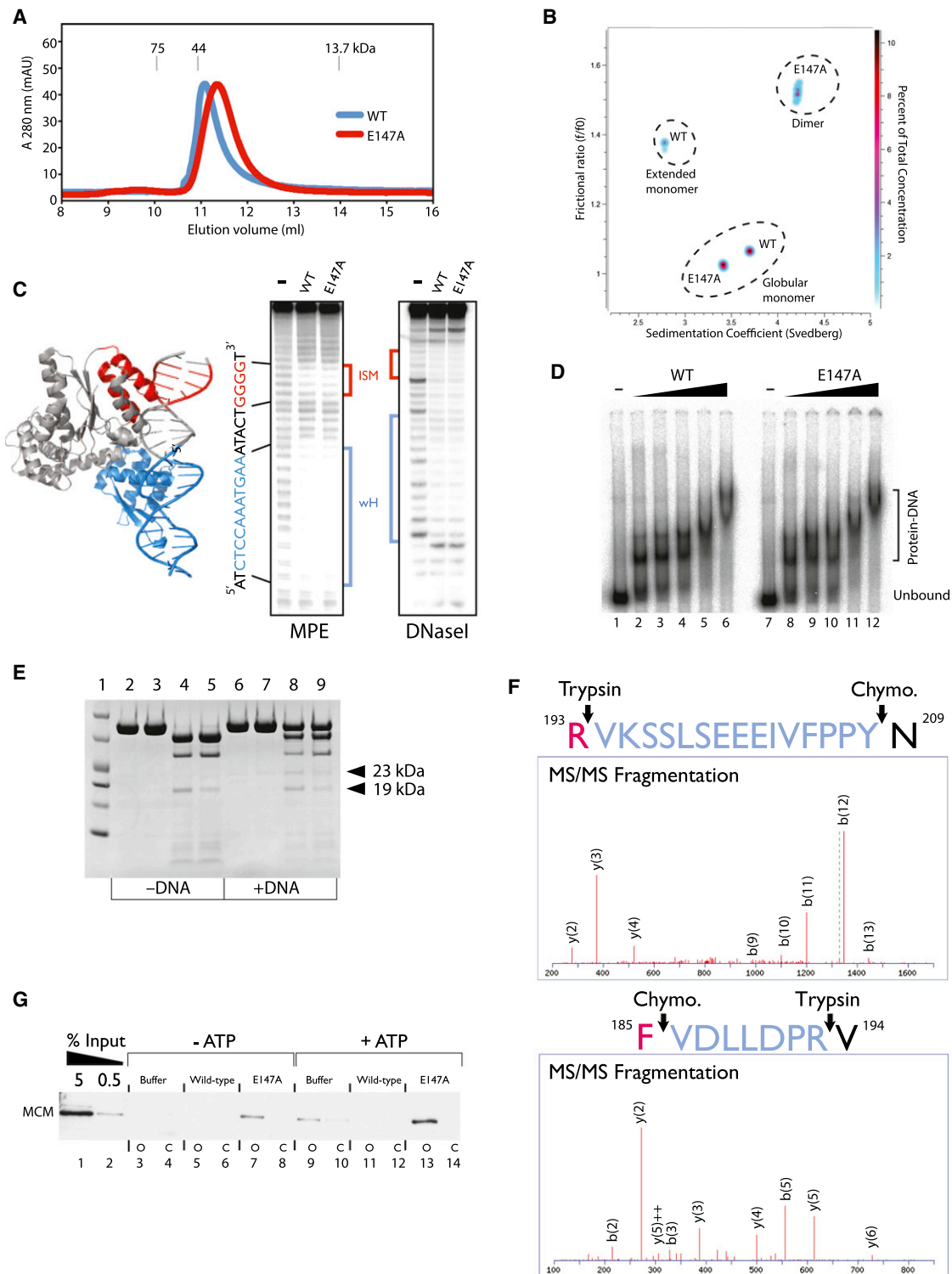


Figure 7. Biochemical Consequences of the Walker B E147A Mutation

(A) Nucleotide-dependent conformational changes in Orc1-1. Gel filtration analysis of 10 μ g of WT Orc1-1•ADP or the E147A•ATP on a Superdex 75 column. The elution positions of size standards are indicated (conalbumin, 75 kDa; ovalbumin, 44 kDa; and ribonuclease A, 13.7 kDa).

(B) Composite three-dimensional contour plot of hydrodynamic parameters obtained from GA-MC50 analysis of WT and Walker B mutant Orc1-1 (E147A). WT analysis reveals the presence of two conformers of the monomer, while the Walker B mutant results indicate the presence of a single monomer conformer in addition to a dimer form (see also Figure S3).

(legend continued on next page)

a distinct clade of Orc1/Cdc6s; nevertheless, our AUC and gel filtration data suggest that the identity of the nucleotide bound by Orc1-1 also modulates the conformation of this protein in solution.

Superimposition of the ADP-NP-bound Orc1-2 structure onto the Orc1-1•ADP-DNA structure, aligning via the principal DNA-binding wH domain, suggests three possible consequences of ATP binding (Figure S4). First, the ISM could disengage from the DNA, conceivably allowing the AAA+ domain to be accessible for interaction with another protomer or client protein; second, both wH and ISM could remain engaged with DNA with consequent remodeling of the intervening duplex DNA; third, both domains remain engaged with DNA and the protein undergoes an internal conformational alteration without modulating DNA structure. To test for the first two scenarios, we performed methidium propyl EDTA footprinting with the WT and E147A proteins on an isolated DNA binding site, the ORB2 element. This technique allows clear resolution of the DNA sequences contacted by wH and ISM. As can be seen from Figure 7C, no discernable difference is seen between the protection patterns mediated by the two proteins, indicating no significant disengagement from, or remodeling of, the DNA binding site. Similarly, there was no discernable difference in the protection pattern upon DNaseI digestion. The same result was observed with ORB1 and ORB3 DNA (data not shown). Furthermore, electrophoretic mobility shift assay (EMSA) analysis with full-length origin DNA fragments (Figure 7D) reveals no significant changes in the affinity for DNA or pattern of shifted complexes generated by either the Orc1-1•ADP or the Orc1-1 E147A•ATP forms of the protein. Additionally, we could not detect melting of origin DNA, by either potassium permanganate or nuclease P1 sensitivity, by either protein in the presence or absence of nucleotide (data not shown). In light of our AUC and gel filtration data revealing altered conformations of the monomeric protein, we therefore speculated that the principal effect of ATP binding was on the conformation of the protein itself rather than on that of the DNA. To investigate this further, we subjected WT and Walker B mutant Orc1-1 to limited proteolysis with trypsin in the presence and absence of saturating levels of ORB2 DNA. Under both conditions, we observe reduced levels of a ~19 kDa species with the Walker B mutant form. In the presence of DNA, the digest patterns of both forms of the protein are altered, with a novel 23 kDa species appearing (Figure 7E). This species

was also more abundant with the WT protein. We recovered the gel bands, digested them with chymotrypsin, and subjected the resultant peptides to mass spectrometry (Figure 7F). The upper band yielded two peptides that corresponded to the product of trypsin digestion at R193 and chymotrypsin digestion at Y208 or F185. This is in good agreement with the apparent size of the species of ~23 kDa (trypsin cleavage at R193 would generate a C-terminal fragment of 22.8 kDa and a N-terminal fragment of 22.5 kDa). The ~19 kDa band also yielded a peptide derived from trypsin cleavage at R193 and chymotrypsin at F185. Thus, the ATP-bound form of Orc1-1, both in DNA-bound and unbound form, displays altered sensitivity of R193 to trypsin digestion. Significantly, R193 corresponds to the arginine finger of the protein's AAA+ site. Thus, in the absence of any observable alterations to the affinity, geometry, or stoichiometry of the protein-DNA interaction between Orc1-1 and DNA, we propose that the differential sensitivity to trypsin reflects a conformational alteration of the ATP-bound form of the protein on DNA that renders it permissive to recruitment of the MCM helicase.

Finally, we established an *in vitro* assay for MCM recruitment to *oriC1*. In this assay, we immobilized origin or nonorigin DNA on paramagnetic beads, preincubated with WT (ADP-bound) or E147A Orc1-1 in the presence or absence of supplemental ATP and then added cell extract prepared from logarithmically growing *S. islandicus* cells. Following incubation at 50°C and washing, beads were recovered and bound proteins eluted by boiling in SDS-PAGE loading buffer prior to western blotting. As can be seen in Figure 7G, neither extract alone nor extract supplemented with WT (ADP-bound) Orc1-1 supports significant MCM recruitment in the absence of ATP. Extract with additional ATP supports recruitment, but interestingly this recruitment is lost by the addition of 1 μM WT Orc1-1•ADP, presumably as a consequence of competition with endogenous Orc1-1 for the 200 pM template. Importantly, however, the E147A mutant supported robust, ATP-stimulated recruitment of MCM in these assays.

DISCUSSION

Taken together, our data yield insight into the evolution, regulation, and mechanism of replication initiation in the chromosome of *Sulfolobus* species. We find that the chromosome is a mosaic

(C) The structure of the *Aeropyrum pernix* Orc1-1 ortholog bound to an ORB element from *oriC1* is shown. The ISM is shown in red and the wH domain in blue. (Figure generated using Pymol from Protein Data Bank file 2V1U.) DNA contact points are correspondingly color-coded. The structure is aligned to the sequence of the *S. islandicus oriC1* ORB2 element. The left-hand gel image shows Fe•MPE cleavage patterns in the absence (–) or presence of 1 μM WT or Walker B E147A mutant protein (WB) in the presence of 2 mM ATP (WB). Positions of protection from cleavage are identified at the right of the panel. The right-hand panel shows DNaseI footprinting performed in the absence (–) or presence of 1 μM WT or Walker B E147A mutant protein (WB) in the presence of 2 mM ATP (WB).

(D) EMSA analysis of WT or Walker B E147A mutant protein binding to a 231 nt probe containing *S. islandicus oriC1*. Lanes contain 0, 63, 125, 250, 500, or 1000 nM of the indicated Orc1-1 protein.

(E) Trypsin cleavage products following 22 hr digestion of WT (lanes 2, 4, 6, and 8) and Walker B mutant (lanes 3, 5, 7, and 9) in the absence (lanes 2–5) or presence (lanes 6–9) of ORB2 DNA.

(F) Identity of the terminal trypsin digestion site by determined by liquid-chromatography tandem mass spectrometry.

(G) Western analysis of MCM recruitment to paramagnetic beads containing either control DNA (c) or *oriC1*-containing DNA (o). Reactions contained DNA at 200 pM and were preincubated with buffer (lanes 3, 4, 9, and 10) or 1 μM WT recombinant protein (lanes 5, 6, 11, and 12) or Walker B mutant E147A Orc1-1 (lanes 7, 8, 13, and 14) in the presence (lanes 9–14) or absence (lanes 3–8) of 2 mM ATP. Following preincubation, extract was added, incubated, and then washed prior to elution of bound proteins by boiling with SDS PAGE loading buffer. 5 and 0.5% input extract were loaded in lanes 1 and 2. MCM was detected by western blotting. See also Figure S4.

of three distinct replicons, each with its own principal initiator protein. In particular, we note that initiation at *oriC3* is not impacted upon by individual or combined *Orc1/Cdc6* deletions but is abrogated by loss of expression of the *Cdt1* homolog *WhiP*. Intriguingly, parallels can be drawn with metazoa: in *Drosophila*, the origin recognition complex (ORC) is dispensable for endoreduplication, whereas *Cdt1* is essential for this process (Park and Asano, 2008). We note that it was not possible for us to generate a triple *Orc1* mutation in which all three paralogs were deleted. Given that *Orc1-2* does not bind in vivo to any origin, we speculate that this synthetic lethality may reflect nonorigin roles for the *Orc1/Cdc6* proteins. Indeed, our ChIP-seq analyses reveal multiple nonorigin genomic binding sites for the *Orc1/Cdc6* proteins. Furthermore, the single mutants show significantly altered transcript profiles compared to WT cells (R.Y.S. and S.D.B., unpublished data). Thus, as with *DnaA* and eukaryal ORC proteins (Scholefield et al., 2011), it appears that archaeal *Orc1/Cdc6* proteins play roles in transcriptional regulation.

We speculate that the mosaic nature of the *Sulfolobus* chromosome has arisen by integration of extrachromosomal elements into an ancestral chromosome that presumably contained a single origin of replication. Given the broad conservation of *Orc1-1* orthologs and their DNA binding sites across the archaeal domain of life (Robinson et al., 2004), we suggest that the ancestral chromosome was dependent on *Orc1-1* and *oriC1*. Because *WhiP* homologs are found in the *Desulfurococcales* and *Sulfolobales* and *Orc1-3* is restricted to the *Sulfolobales*, we propose the sequential acquisition of first *WhiP/oriC3* prior to the bifurcation of these two lineages, followed by integration of the *Orc1-3/oriC2* replicon in the branch leading to the *Sulfolobales*. Interestingly, the genome replication mode of the bacterium *Vibrio cholera* also appears to have been sculpted by acquisition of extrachromosomal element replication origins. However, in that organism, the genome is split between two chromosomes, each with a single origin: one dependent on a classic bacterial *oriC/DnaA* system and the second having an origin with features reminiscent of plasmid origins. Indeed, this partitioning of bacterial genomes into multiple single-origin chromosomes is found in a range of bacterial species (Egan et al., 2005; Egan and Waldor, 2003). In contrast, multiple-origin single-chromosome genomes are found in a number of archaea. Why are archaeal chromosomes permissive in this regard? One explanation may lie in the contrasting mechanisms for replication termination in bacteria versus archaea (Duggin et al., 2008b; Toro and Shapiro, 2010). Whereas termination in bacteria involves polar fork traps, we have recently found that termination in *Sulfolobus* is by random fork collision (Duggin et al., 2011). In support of this, our MFA data on the mutant strains in which only a single origin fires reveal that termination is repositioned to occur 180° across the chromosome from the active origin (Figure 2F). An ectopic origin integrated into one replicore of a bacterial chromosome would result in premature arrival and prolonged stalling of a fork at a termination site. This may impact genome stability. In addition, because of the presence of the fork traps, the time taken to replicate a two-origin bacterial chromosome would still be dependent on the length of time it takes the fork arising from *oriC* to traverse the longest replicore. In contrast, the absence of active termination sites in *Sulfolobus*

would mean that additional origins would simply result in repositioning of termination events to midway between active origins and a concomitant reduction in the total time spent in S phase, assuming similar timing of firing of the origins. The ability to accommodate and regulate multiple origins will have played a major role in permitting the increase in genome size seen in eukaryotic organisms (Diffley, 2011).

Our data also reveal mechanistic insight into the action of the *Orc1-1* initiator. As with bacterial *DnaA*, we find that the ATP-bound form of the initiator is the active form (Kaguni, 2011; Mott and Berger, 2007). Our expression profiling reveals a wave of transcription of the genes for the *Orc1-1* and *Orc1-3* initiators immediately prior to the onset of replication. We propose that this imposes a temporal window for initiation in which the newly synthesized proteins bind to ATP and associates with origin DNA before hydrolysis of ATP to ADP inactivates the proteins. It may, therefore, be significant that *Orc1-1* and *Orc1-3* are encoded in the immediate vicinity of their cognate origins. Furthermore, expression of WT *Orc1-1* driven by its native promoter from episomes, while resulting in origin occupancy (as adjudged by ChIP), fails to activate the origin, presumably due to hydrolysis of ATP in the cell prior to origin binding. However, expression of the Walker B ATP hydrolysis-defective mutant to the same level results in an overreplication phenotype.

In bacteria, the ATP-bound form of *DnaA* facilitates the formation of higher-order structures and is able to mediate localized DNA melting at the origin (Bramhill and Kornberg, 1988; Duderstadt et al., 2011; Erzberger et al., 2006; Katayama et al., 2010; Ozaki et al., 2008). Although we do observe a subpopulation of the Walker B mutant form of the protein existing as dimer in solution, we find that the ADP- and ATP-bound forms of *Orc1-1* show equivalent DNA binding properties. Furthermore, we have been unable to observe any localized sensitivity to nuclease P1 or potassium permanganate, indicative of DNA melting, induced by either form of the protein (data not shown). Perhaps unsurprisingly, in this regard *Orc1-1* is behaving in a manner more reminiscent of eukaryal ORC. There is no evidence for eukaryal ORC melting origins; rather, the available data support a model whereby MCM(2-7) is recruited to double-stranded DNA with duplex unwinding occurring at a later stage (Evrin et al., 2009; Heller et al., 2011; Remus et al., 2009). We propose that this is an evolutionarily conserved feature of the *Orc1/Cdc6* proteins and that these proteins act principally to define loci for MCM recruitment. In light of our protease sensitivity assays, we suggest that ATP acts to modulate the conformation of *Orc1/Cdc6* proteins on the DNA binding site such that the protein is able to effectively recruit MCM. Subsequent hydrolysis of ATP to ADP by *Orc1/Cdc6* then renders the protein incapable of recruiting further MCM, thereby contributing to the fidelity of replication control. This simple binary switch model of archaeal *Orc1/Cdc6* function is supported by our observation of the overreplication phenotype mediated by the Walker B mutant of *Orc1-1*. We note that in budding yeast, temporally distinct ATP binding and hydrolysis events by both the ORC complex and *Cdc6* are required for iterative MCM(2-7) loading at replication origins (Randell et al., 2006). The increased organizational complexity of the eukaryotic origin definition and MCM recruitment system is likely a consequence of the increased regulatory demands

imposed by the large number of replication origins found in eukaryotic chromosomes (Diffley, 2011). The simple binary-switch model that we propose for Orc1/Cdc6 function is clearly distinct from the mechanism of action of the bacterial initiator DnaA and provides a paradigm for the function of initiator proteins in archaea and eukaryotes.

EXPERIMENTAL PROCEDURES

Strains, Media, and Growth Conditions

S. islandicus strain E233S (Δ pyrEF Δ lacS) constructed previously (Deng et al., 2009) was used as the host for genetic manipulations. All archaeal strains used in this work, including the genetic host and its transformants of diverse plasmids as well as the *orc1/whip*, are summarized in Table S1. These archaeal cell lines were grown in SCVY (0.2% sucrose, 0.2% Casamino acids, 5 ml vitamin solution, and 0.005% yeast extracts) or TYS (0.1% tryptone, 0.05% yeast extracts, and 0.2% sucrose) medium at 78°C. If required, uracil was supplemented to 20 μ g/ml while 5'-fluoroorotic acid (5'-FOA) was added to 50 μ g/ml, in SCVY medium. Microscopy was performed as described previously (Samson et al., 2011).

Protein Purification and Biochemical Analyses

Orc1/Cdc6 and WhiP proteins were expressed in *E. coli* with C-terminal Hexa-His tags and purified as described previously (Robinson and Bell, 2007; Dueber et al., 2007). EMSAs and footprinting were performed as described previously (Dueber et al., 2007; Robinson et al., 2004). The MCM recruitment assay was based on the approach initially established by Seki and Diffley (2000). A total of 10 μ g of a 5 kb plasmid (pCR Script containing a 2 kb sequence centered around *oriC1* or a control locus, *Sire_2328*) was linearized by EcoRI and PstI digestion, the EcoRI-generated recessed end was filled in with Klenow fragment (New England Biolabs) and Biotin-14-dATP and dTTP. The DNA was recovered from the reaction mixture using QiaQuick column (QIAGEN) and immobilized on Dynabeads M-280 streptavidin beads following the manufacturer's recommendations (Life Technologies). Binding reactions were assembled in LoBind tubes (Eppendorf) and contained 200 pM DNA in 50 μ l of binding buffer (20 mM Tris acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM DTT [pH 7.9] at 25°C) plus 20 ng/ μ l poly(dGdC). Reactions were heated to 50°C in a water bath and buffer or Orc1-1 protein (1 μ M final) were added and supplemented with nucleotide to 2 mM as indicated. Following a 10 min incubation, 20 μ l of a 5 mg/ml whole-cell extract of *S. islandicus* was added (see below). Then, 20 μ l of water or supplemental ATP to 2mM as indicated was added and the reaction incubated for a further 30 min at 50°C before washing twice by magnet-mediated bead pelleting and resuspension in 100 μ l of prewarmed binding buffer, followed by one wash in 100 μ l binding buffer containing 500 mM potassium acetate. Following this final wash, beads were resuspended in 1xSDS PAGE loading buffer and subjected to SDS PAGE followed by western blotting. All wash steps employed preheated buffers and were performed in the 50°C water bath. We observed a very high background of recombinant Orc1-1 protein bound to both DNA substrates and even to beads alone (data not shown), but MCM was only efficiently recruited to the origin-containing DNA substrate.

The *S. islandicus* cell extract was prepared by growing cells to midexponential phase (OD 600 nm = 0.4) before harvesting and resuspension in 2x binding buffer (40 mM Tris acetate, 100 mM potassium acetate, 20 mM magnesium acetate, 2 mM DTT [pH 7.9] at 25°C). Cells were lysed using a FrenchPress (Thermo) and the extract was clarified by centrifugation at 23,700 *g* for 15 min at 4°C. Then, 100 μ l aliquots were prepared in LoBind tubes and snap frozen on dry ice. Total protein concentration was determined by Bradford assay to be 5 mg/ml. Extracts were stored at -80°C.

Flow Cytometry

Flow cytometry of *S. islandicus* cells was performed essentially as previously described for *S. acidocaldarius* (Duggin et al., 2008a). Cells were fixed with 70% ethanol and their DNAs were stained with Mithramycin A and analyzed with A-40 Apogee flow cytometer.

Neutral-Neutral 2D Gel Electrophoresis

S. islandicus cells were collected by centrifugation and washed twice with TEN buffer (50 mM Tris-Cl [pH 8], 50 mM EDTA [pH 8], 100 mM NaCl). Cell suspensions were then mixed with low-melting-point agarose and dispensed into Bio-Rad plug molds. Genomic DNA preparation and 2D gel electrophoresis were performed as described previously (Robinson et al., 2004). For further details, please refer to Extended Experimental Procedures.

Chromatin Immunoprecipitation

ChIP was performed as described by Robinson et al. (2004). For further details, please refer to Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and five tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2013.01.002>.

LICENSING INFORMATION

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